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ORIGINAL PAPER

Comparision between *Bacillus subtilis* RP24 and its antibiotic-defective mutants

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Abstract *Bacillus subtilis* RP24, a promising plant growth-promoting rhizobacterium and a potent biocontrol agent isolated from pigeonpea rhizosphere was mutagenized with ethyl methanesulphonate to study the possible mechanism/s involved in the potential antagonistic properties of the strain. Over 10,000 mutants were screened against the phytopathogenic fungus *Macrophomina phaseolina* on potato dextrose agar plates to select ten mutants showing partial antagonism as compared to the parent strain and one negative mutant showing no antagonism. The parent strain RP24 was compared with its mutants for the presence of different possible mechanisms behind antagonism. Production of hydrogen cyanide, ammonia, siderophores, and hydrolytic enzymes like lipase, amylase, and protease were detected in all the mutants as well as the parent strain, whereas fungal cell-wall-degrading enzymes, β-1, 3-glucanase and chitosanase were not detected in any of the mutants and the parent strain, indicating that none of these mechanisms was involved in the antagonistic trait of the strain. Two possible mechanisms detected behind the antifungal trait of the strain RP24 were production of antifungal volatiles and extra-cellular diffusible antibiotics. An attempt was made for extraction, partial characterization of the extra-cellular diffusible antifungal metabolite/s by thin layer chromatography and sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE). The extracellular, methanol soluble, hydrophobic, ninhydrin-negative, thermostable and pH-stable antifungal metabolites were characterized as cyclic lipopeptides belonging to the iturin group of peptide antibiotics.

Keywords *Bacillus subtilis* RP24 · PGPR · Mutagenesis · Antifungal metabolites

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Introduction

The genus *Bacillus* represents the most utilized biocontrol agent because of its inherent capacity to produce resistant endospores, tolerance to heat, cold, pH extremes as well as to pesticides, fertilizers and storage (Pal and Jalali 1998). Several potent strains of *Bacillus* sp. have been identified and tested on a wide variety of plant diseases. Different mechanisms like production of hydrogen cyanide (HCN), ammonia, siderophores, antibiotics and various hydrolytic enzymes like lipase, glucanase, and chitinase (Howell et al. 1988; Voisard et al. 1989; Von Dohren 1995; Trivedi et al. 2008) have been suggested for the biocontrol properties of microorganisms. In the past, the principal mode for biocontrol action of the genus *Bacillus* had been credited with the production of antibiotics (Katz and Demain 1977; Cubeta et al. 1985; Loeffler et al. 1986). In the last decade, production of lytic enzymes like glucanolytic and proteolytic enzymes has been related to the antagonistic properties of *Bacillus* (Nielsen and Sorensen 1997). Some workers have suggested the role of certain unknown volatile compounds produced by some *Bacillus* sp. in

antagonizing cyanobacteria, free-living protozoa, plants, and fungi (Wright and Thompson 1985; Fiddaman and Rossall 1993).

In the present investigation a potent biocontrol agent, *Bacillus* sp. RP24 isolated from pigeonpea rhizosphere (Minakshi 2002; Minakshi et al. 2005) later identified as *Bacillus subtilis* RP24 (unpublished data) was subjected to chemical mutagenesis and the mutants lacking antagonistic traits against the test fungus were selected. The main aim of this study was to identify the exact mechanism/s responsible for the antagonistic trait of the strain. Therefore the parent strain was compared with the mutants for different possible mechanisms that may be responsible for the antifungal trait. An attempt was also made for extraction, partial purification and characterization of the antifungal metabolite/s. Biochemical characterization of the parent and mutant strains was also done to ascertain the physiological changes in the strain after mutagenesis.

Materials and methods

Micro-organisms and culture conditions

Bacillus subtilis RP24 the strain employed in this study was maintained on tryptone yeast extract (TY) agar medium under refrigerated conditions and raised in TY broth as and when required. The indicator soil-borne pathogenic fungi *Macrophomina phaseolina* was procured from the Indian Type Culture Collection, Indian Agricultural Research Institute, New Delhi, India and was maintained and multiplied on potato dextrose agar (PDA) medium.

Induction, selection, and characterization of bacterial mutants

Mutagenesis using ethyl methanesulphonate (EMS; 1.206 g/ml at 20°C, Sigma–Aldrich) was carried out by the procedure described by Miller (1992). *Bacillus subtilis* RP24 cells were grown in 30 ml TY medium to mid-log phase, harvested, washed and resuspended in 15 ml of minimal A buffer containing (/l) 15.5 g K_2HPO_4, 4.5 g KH_2PO_4, 1 g $(NH_4)_2SO_4$ and 0.5 g sodium citrate·$2H_2O$. The suspended cells were allowed to grow with shaking at 30°C and 2 ml samples were withdrawn and treated with different concentrations (0, 5, 15, 25, 40, 50 µl/ml) of EMS for different incubation periods (30 and 60 min). Following treatment, the cells were washed twice with minimal A buffer and resuspended in 2 ml of same buffer. Viable cell counts corresponding to each concentration and incubation period were determined by dilution plating of cells on TY agar medium and compared with that of corresponding control (without EMS) treatment. The treatments showing over 99.9% killing were selected and the individual mutants were stored as glycerol stock at −20°C for further use. For selection of antifungal negative mutants, screening of the mutants was done against *M. phaseolina* by dual culture method on PDA plates. The mutants lacking antagonism (partially or completely) against the test pathogen were selected and maintained on PDA slants.

The parent and the mutant strains were compared for the production of HCN on King's B medium containing glycine (Bakker and Schippers 1987), ammonia in peptone water (Dey et al. 2004), siderophores on chrome azurol S (CAS) agar medium (Schwyn and Neilands 1987) and volatile antifungal metabolites (Fiddaman and Rossall 1993). The production of hydrolytic enzymes, chitosanase (Ohtakara 1988) and β-1, 3-glucanase (Mulano et al. 1977) was tested by using glycol chitosan and laminarin as substrates, respectively and subsequently estimating the released reducing sugars by the DNS method (Miller 1959).

Morphological and biochemical properties of the parent and mutant strains were compared by conducting phenotypic tests like colony morphology and motility and biochemical tests like Gram staining, catalase, oxidase, hydrolysis of starch, casein, urea, arginine, gelatin and lipid, fermentation of sugar, triple sugar-iron agar test, nitrate reduction, litmus milk test, citrate utilization, hydrogen sulfide production, Voges Proskauer test, indole production, fluorescent pigment production, glucose and lactose utilization and presence of poly-β-hydroxybutyrate by following *Bergey's Manual of Determinative Bacteriology* (Holt 1994).

Extraction and partial characterization of antifungal metabolite/s

For extraction of antifungal metabolite/s the parent and mutant strains grown in potato dextrose broth were centrifuged at 8000*g* at room temperature. The supernatant was extracted twice with ethyl acetate after adjusting the pH to 2.0 with 6 M HCl. The ethyl acetate fractions were pooled and evaporated on a rotary evaporator at 60°C. The dried fractions were dissolved in minimum quantity of methanol and were bioassayed against *M. phaseolina* on PDA plates by a well diffusion method. The metabolites in the methanol fraction were separated by preparative TLC, using chloroform:methanol:ethanol:water (70:30:35:5) solvent system as mobile phase. The spots on the plates were developed with iodine vapour and R_f values were recorded.

Ethyl acetate extracts of the parent and negative mutant strain were extracted with different non-polar and polar solvent systems for partial purification of the metabolite/s. These partially purified fractions suspended in an equal

volume of methanol were filtered through a 0.22 μm Millipore membrane and bioassayed against *M. phaseolina*. Equal volumes (100 μl) of the active fraction of parent strain and the corresponding fraction of the negative mutant were loaded on 15% polyacryamide gel containing sodium dodecyl sulphate (SDS) and the electrophoresis was carried out at 34 mA for 7 h followed by staining with Coomassie Blue R250.

pH and thermal stability of antifungal metabolite/s

For the thermostability test the filter-sterilized crude extract was exposed to different temperature (60, 80, and 100°C) for 30 min and also autoclaved at 121°C for 20 min. The remaining antifungal activity of the extract was assayed against *M. phaseolina* after cooling it to room temperature. To test the effect of pH, the extract was adjusted to pH values in the range 1.0–14.0 separately using either 0.5 M HCl or NaOH and incubated for 24 h at 20°C. The samples were restored to neutral pH and subjected to bioassay.

Results and discussion

Selection of mutants and comparision with the parent strain

Treatments of EMS at concentrations of 40 and 50 μl/ml for 60 min resulted in more than 99.9% killing of the cells, hence these were selected (Table 1). Of the 10,000 mutants screened against, 10 mutants (PM1 to PM10) showed partial inhibition of the test fungus as compared to the parent strain whereas one mutant (NM) completely lacked the ability to inhibit the fungal growth on PDA plate (Figs. 1, 2).

All the mutants and the parent strain could produce siderophores, HCN and ammonia (Table. 2) in vitro. Production of fungal cell-wall-degrading enzymes, chitosanase and β-1, 3-glucanase was not observed in any of the mutants and the parent strain, whereas production of hydrolytic enzymes viz. lipase, protease and amylase was detected in all the mutants as well as the parent strain

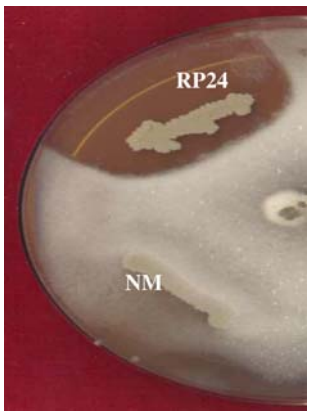

Fig. 1 Bioassay of RP24 and its negative mutant against *M. phaseolina*

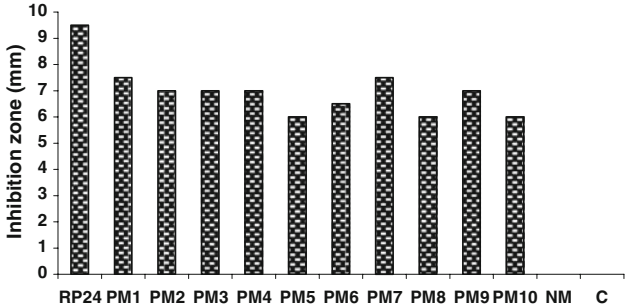

Fig. 2 Antagonism exhibited by RP24 and its mutants against *M. phaseolina*

indicating that none of these mechanisms was related to the biocontrol properties of the strain RP24. Earlier workers have correlated the production of glucanolytic and proteolytic enzymes with antagonism in *Paenibacillus polymyxa*, *B. pumilis* and *Bacillus* sp. (Nielsen and Sorensen 1997). The production of HCN, siderophores, ammonia, lipase and chitinase in growth medium by *Pseudomonas corrugata* was considered contributing to the antagonistic activity of the bacterium against *Alternaria alternata* and *Fusarium oxysporum* (Trivedi et al. 2008). It is less likely that the above proposed mechanisms be involved in antagonistic behaviour of the strain, instead we believe that some other mechanism/s might be operating behind antagonism. This could be further supported in our observation on

Table 1 Action of EMS on *Bacillus subtilis* RP24

Concentration of EMS (μl/ml)	c.f.u./ml	Mortality (%)	c.f.u./ml	Mortality (%)
0	3.0×10^8	00.000	3.5×10^8	00.000
5	2.9×10^8	3.333	3.3×10^8	0.571
15	2.2×10^8	26.667	2.0×10^8	42.875
25	6.5×10^7	78.333	1.5×10^7	95.714
40	4.7×10^7	84.333	1.3×10^5	99.963
50	2.2×10^7	92.666	6.0×10^4	99.983

production of antifungal volatiles (that could inhibit radial growth of *M. phaseolina*) by the parent strain and not by the negative mutant. Among the partial mutants, production of antifungal volatiles was observed only in four strains (PM1, PM2, PM7, and PM9) whereas six strains (PM3, PM4, PM5, PM6, PM10; Table 2) could not produce antifungal volatiles, indicating the presence of additional mechanism/s for antagonism other than the production of volatile compounds.

Morphologically the parent, negative mutant and partial mutant (except PM4) strains were identical and formed cream coloured, opaque, wrinkled and spreading colonies on TY medium. Partial mutant PM4 produced light brown coloured colonies, probably due to the production of a non-diffusible brown-coloured pigment. Microscopic studies revealed the presence of rod-shaped, motile cells arranged in chains for all the strains. Formation of a central spore was observed in the parent strain, partial mutants as well as in the negative mutant. Spore formation has been correlated with antibiotic production in *Bacillus* (Demain 1974; Hanson et al. 1970). Peptide antibiotics might be used in several ways by an organism during the process of sporulation as modifiers of the cell membrane (Hodgson 1970) but they are not necessarily related to sporulation as indicated by the ability of antibiotic-negative mutants of *B. subtilis* and *B. licheniformis* to sporulate normally (Haavik and Froyshov 1975). Further all the strains behaved in a similar way for all the biochemical tests conducted (Table 3) except for two tests. The parent strain and partial mutants showed positive results for urease production and Voges Proskauer test whereas the negative mutant strain responded negatively to these two tests, thus indicating the effect of mutagenesis on biochemical properties of the negative mutant.

Extraction and partial characterization of diffusible antifungal metabolite/s

The crude ethyl-acetate extracts of the parent and the mutant strains were bioassayed against *M. phaseolina* on PDA plates by well diffusion. Extracts of partial mutants showed partial inhibition of the radial growth of the test pathogen as compared to that of parent strain, whereas extract of the negative mutant could not inhibit the radial growth of the test pathogen (Fig. 3). These results confirmed the production of extracellular diffusible antifungal compounds by the parent strain as well as the partial mutants. Thus it can be proposed that volatile as well as diffusible metabolites are responsible for the antagonistic trait of the *Bacillus subtilis* RP24. Chaurasia et al. (2005) isolated an efficient strain of *Bacillus* from tea rhizosphere that could produce both volatile and diffusible antifungal compounds. The inhibitory effect caused by volatiles was greater than that of diffusible compounds. Further the inability of the negative mutant to produce any of the diffusible or volatile antifungal metabolite indicates some sort of linkage between the two mechanisms.

The crude extract was stable and exhibited 100% activity after exposure to 60, 80, and 100°C for 30 min whereas 20% of the activity was lost on autoclaving at 121°C for 20 min. Further the extract exhibited stability over a wide range of pH values from 4.0 to 10.0. At acidic pH (3.0) the precipitation started and the activity was completely lost at pH 2.0. These features grant metabolite/s the potency and flexibility for formulation, production and pharmaceutical applications.

Thin layer chromatography of the crude extracts of parent and the mutant strains showed many spots throughout the chromatogram after iodine vaporization.

Table 2 Comparision of parent and mutant strains for the presence of possible mechanism/s behind antagonism

Strain	Siderophores	HCN	NH_3	β 1,3-glucanase	Chitosanase	Lipase	Volatile antifungals	Diffusible antifungals
RP24	+	+	+	−	−	+	+	+
NM	+	+	+	−	−	+	−	−
PM1	+	+	+	−	−	+	+	+
PM2	+	+	+	−	−	+	+	+
PM3	+	+	+	−	−	+	−	+
PM4	+	+	+	−	−	+	−	+
PM5	+	+	+	−	−	+	−	+
PM6	+	+	+	−	−	+	−	+
PM7	+	+	+	−	−	+	+	+
PM8	+	+	+	−	−	+	−	+
PM9	+	+	+	−	−	+	+	+
PM10	+	+	+	−	−	+	−	+

Table 3 Morphological and biochemical characterization of parent and mutant strains

Morphological and biochemical tests	RP24	Partial mutants	NM
Shape and arrangement of cells	Rods in chains	Rods in chains	Rods in chains
Spore formation	+	+	+
Gram reaction, motility, catalase, nitrate reduction, lipid hydrolysism, gelatin hydrolysis, casein hydrolysis, starch hydrolysis, lysine de-carboxylation, spore formation, glucose utilization, presence of PHB	+	+	+
Oxidase, citrate utilization, complete denitrification, pigment production, H_2S production, methyl red, arginine dihydrolysis, lactose utilization	−	−	−
VP-test, urea hydrolysis	+	+	−

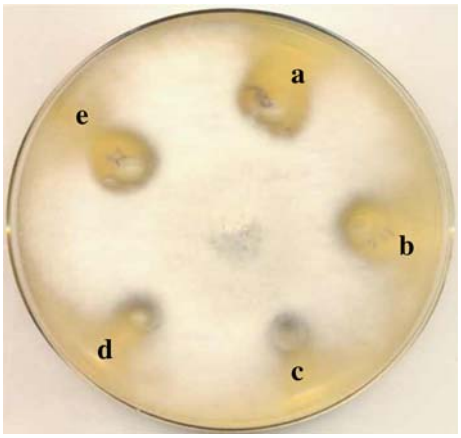

Fig. 3 Bioassay of ethyl acetate extracts of RP24 (*a*) and its mutants (*b* PM1, *c* NM, *d* PM3, *e* PM9) against *M. phaseolina*

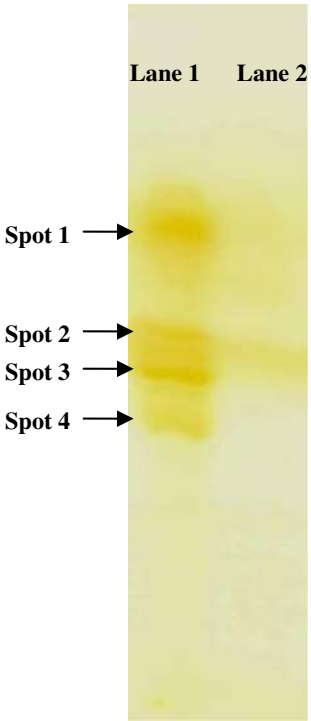

Fig. 4 Thin layer chromatography of partially purified extract of RP24 (*lane* 1) and its negative mutant NM (*lane* 2)

But the negative mutant lacked the two spots at R_f 0.71 and 0.58 that were present in all other samples, indicating the possible role of these spots in antagonism (Fig. 4).

The crude extracts of the parent and negative mutant strains were further fractionated using different solvents (data not shown) for partial purification of the antifungal metabolites. The chromatogram of the partially purified methanolic fractions showed the presence of four spots for the extract of the parent strain with R_f values of 0.71, 0.65, 0.62, and 0.58, whereas no band was produced by the corresponding fraction of mutant strain (Fig. 4). The bands responded negatively to ninhydrin, indicating the absence of free amino acids and produced white spots when sprayed with water, thus indicating the hydrophobic nature of the metabolites. All the spots could inhibit the test pathogen independently, with spot no. 4 (R_f 0.58) showing maximum activity followed by spot 3 (R_f 0.60). The spot no. 1 (R_f 0.71) showed minimum inhibition potential. These results indicate the presence of four active antifungal metabolites. Partially purified methanol fractions when run on 12.5% acrylamide gel in SDS–PAGE showed one band with molecular weight between 1.0–1.5 kDa whereas no band was observed for the negative mutant establishing the proteinaceous nature of the compound. The appearance of only one band in the methanolic extract of parent strain in SDS–PAGE suggested that the peptide antibiotics produced by the organism may be closely related and they may have similar molecular masses. Bechard et al. (1998) isolated an antimicrobial lipopeptide from *Bacillus subtilis* which showed molecular weight ~1.5 kDa on SDS–PAGE (Fig. 5). Wu et al. (2005) isolated two peptide antibiotics from *B. subtilis* with molecular weight 1,422.71 and 1,422.65 Da. The genus *Bacillus* is known to produce lipopeptide antibiotics that are smaller than most proteins. A family of closely related peptides rather than a single substance said to be produced by this organism. The lipopeptide antibiotics often contain unique amino acids and are

Fig. 5 SDS–PAGE of partially purified extract of RP24 (*lane* 1) and its negative mutant NM (*lane* 2)

often cyclic (Katz and Demain 1977). We also observed the presence of four extracellular, methanol soluble, hydrophobic, ninhydrin negative antifungal metabolites that were thermo-stable and pH stable. All these properties indicated that these antifungal compounds may belong to iturin group of cyclic lipopeptide antibiotics (Bernal et al. 2002; Chitarra et al. 2003).

Further investigation by HPLC, LC/MS and NMR is needed to determine the chemical structure of the antifungals produced by *Bacillus subtilis* RP24.

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